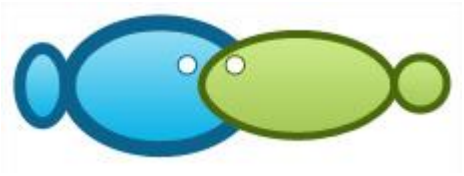

Toxicological effect and acetylcholinesterase (AChE) expression in golden apple snail (*Pomacea canaliculata*) after exposure to chlorpyrifos

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Abstract. Chlorpyrifos, an insecticide, has been extensively used in Thailand, resulting in their accumulation in the environmental compartments: soil, water, air and organisms (including snails). The purpose of this study was to evaluate the effect of chlorpyrifos, in a commercial solution form, on golden apple snail (*Pomacea canaliculata*). Toxicity, acetylcholinesterase (AChE) response, and the median lethal concentration (LC50) were assessed. Commercial chlorpyrifos was diluted with distilled water at the ratio of 1:100,000 (V/V). The results showed that as the exposure time and concentration, so did the accumulative mortality. LC50 values at 24, 48, 72 and 96 h were 7.00 (6.02-8.93), 5.91 (5.29-6.97), 4.90 (4.58-5.41) and 4.22 (4.02-4.50) mL L^{-1}, respectively. The AChE response was assessed in 4 sub-lethal concentration levels (0.5, 1.0, 1.5 and 2.0 mL L^{-1}) by using dot blot and western blot. In the concentration of 2.0 mL L^{-1}, AChE response was detectable only at 24 and 48 h after exposure. The molecular weight of AChE was 71 kDa. The immunohistochemistry showed strong localization of AChE in intestinal tissue, consistent with dot blot and western blot results. Moreover, organophosphate was identified in the water in three locations evaluated for pesticide exposure in *P. canaliculata* using AChE as a bioindicator from Huai-Saneng reservoir, Surin province. These areas are used for agriculture and aquaculture, when locations near a community have no indication of contamination of organophosphorus insecticide. Based on the study, AChE response can potentially be used as a biomarker for indicating chlorpyrifos exposure and *P. canaliculata* could be used as a bioindicator species for aquatic environments.

Key Words: biomarker, chlorpyrifos, insecticides, toxicity testing.

Introduction. Wastewater from many activities including agriculture, habitation, and industry can contain contaminants and toxicants. Particularly, many agricultural areas leach pesticides into the environment, causing adverse effects on both target and non-target organisms in terrestrial and aquatic environments (Magar & Afsar 2013; Grung et al 2015; Georgieva et al 2018). Moreover, Kaur & Sinha (2019) reported that agrochemicals, especially pesticides, pose a considerable threat to aquatic life, due to the runoff which enters into nearby lakes, rivers and streams. The effect of pesticides must be investigated, and many studies were conducted all over the world (Khalil 2015), e.g. the El Temsah and Bitter Lakes of the Suez Canal, Egypt (Said et al 2013), the Tono Reservoir located near the Tono Irrigation Project at Navrongo in Northern Ghana (Akoto et al 2016), Huai−Saneng Reservoir, Surin Province, Thailand (Thanomsit et al 2018), East Lake Wuhan, China (Cui et al 2015) and irrigation sites in the Upper East Region of Ghana (Abah et al 2020).

Chemical insecticides are widely applied for controlling pests. Many assay methods and risk assessments have been developed and applied to insecticides, including

chlorpyrifos. In Thailand, chlorpyrifos (O, O-diethyl-O-3, 5, 6-trichlor 2-pyridyl phosphorothioate; CPF) has been widely used to control foliar insects, affecting agricultural crops and subterranean termites, since it was first introduced into the marketplace in 1965. It is the second largest selling organophosphate in the world, being more toxic to fish than organochlorine compounds (Deb & Das 2013). Banaee (2012) stated that most pesticides also affect non-target species, e.g. aquatic organisms. Besides, the use of insecticides contaminate waters and accumulate in aquatic organisms such as freshwater fishes and snails. Hence, the evaluation of contaminants and their effects are of the utmost importance.

The bioassay principle is mostly used to evaluate the concentration or toxicity of substances, such as insecticides, based on their effects on plant or animal tissues. For an effective bioassay, the indicator species should be sufficiently sensitive to allow detection of small amounts of the substance, as well aș to have an increased response to higher concentrations of the substance. The toxicity of an insecticide depends on its dosage. The median lethal dose (LD50) is usually used for studying the toxicity of an insecticide to an organism. This value indicates the dose per weight unit (commonly expressed as mg kg^{-1}) that is lethal to 50% of the organisms being studied. Similarly, the median lethal concentration (LC50) is the concentration of a compound in the external media (such as air or water), which kills half of the test population. The LC50 is preferred when an exact dose is impractical to give to the organism, small insects for example (Paramasivam & Selvi 2017).

Many methods have been established to determine pesticide contamination in organisms, most of them requiring skilled personnel or complex sample preparation e.g. extraction or purification. If the concentration level in the aquatic environment is below the detection limit of instruments then a biomarker can be a more useful indicator of environmental risk. It can be used to detect the exposure to pesticides and their effects (Chitmanat et al 2008). Acetylcholinesterase (AChE) can be used as a biomarker because it plays an important role in degrading one of the important neurotransmitters, acetylcholine (ACh), in the synaptic cleft. For substrate preference, AChE hydrolyzes ACh rather than other choline esters and exhibits substrate inhibition in high concentration. There are two types of cholinesterase: AChE and butyrylcholinesterase (BuChE) (Yaqin, 2010). Sturm et al (1999) indicated that the difference of AChE and BuChE was the binding of substrates and inhibitors. AChE has been chiefly used as a biomarker in many organisms and can be classified as a specific biomarker of response to organophosphorus pesticide exposure (Walker et al 2006).

Organophosphorus pesticides are insecticides widely used in all parts of Thailand. They cause acute effects by inhibiting the function of AChE, which is a nervous system enzyme (Panuwet et al 2012). This inhibition can be measured to detect exposure of organophosphorus pesticides (Walker et al 2006). The examples of organophosphorus pesticides used in Thailand are chlorpyrifos, dichrotofos, parathion methyl, and profenofos (Panuwet et al 2012). AChE activity is commonly used as an indication of exposure to organophosphorus pesticides for many invertebrate species, including blue mussel (*Mytilus edulis*), fresh water snail (*Lanistes carinatus*) and *Mytilus galloprovincialis* (Van Erp et al 2002; Yaqin 2010; Panuwet et al 2012; Khalil 2015). Thanomsit et al (2018) reported that Surin province, Huai-Saneng reservoir is very important to local residents for both food security and the local economy. Fishing in that reservoir can be performed all year round and the water supplies all agricultural needs in the surrounding area. Therefore, insecticides and herbicides from the organophosphorus group that leach into the reservoir may accumulate and pass through the food chain to the local people.

The golden apple snail, *Pomacea canaliculata* (Orbigny), a species in the family of Ampullariidae (Pilidae), is found in South America, Central America, the West Indies and the southern states of USA. However, they can also be widely found in Southeast Asia, including Thailand, because of their ability to reproduce and spread in a short time period (Banpavichit et al 1994) through rivers, canals and paddy fields. As such, it is likely to be exposed to several agricultural chemicals, so it is expected to be a good bioindicator species. Shellfish, such as *P. canaliculata*, make good bioindicators because of their slow

movement compared to other species, such as fish. This study aimed to evaluate the toxicity of chlorpyrifos on *P. canaliculata* found in aquatic environments, and to investigate any morphological alteration, and localization of AChE in response to chlorpyrifos exposure in sublethal concentrations. The study also evaluated environmental pesticide exposure in *P. canaliculata* from Huai-Saneng reservoir, Surin province using AChE expression as an indicator.

Material and Method

Sampling and acclimatization. Samples of mature *P. canaliculata* were collected from paddy fields in the Mueang district, Surin province, Thailand, in January, 2020. Afterwards, they were acclimatized in a pond in the Department of Fisheries, Faculty of Agriculture and Technology, Rajamangala University of Technology Isan Surin Campus, for 14 days. Their average weight was 20.2±2.1 g with a length of 3.9±0.4 cm and a width of 2.8±0.41 cm. The animal use license number is UI-03405-2559. All procedures involving animals were approved by the committee for biological experimentation on animals from Department of Fishery Science, Rajamangala University of Technology Isan Surin Campus.

Acute toxicity evaluation (Median lethal concentration; LC50) by Probit analysis. Toxicity evaluation was performed in 20 L plastic tanks that were divided into 5 treatments (triplicates were performed for each treatment). The water that filled each tank was prepared by mixing the commercial soluble chlorpyrifos (chorpyrifos 40% weight by volume Emulsifiable Concentrate-EC) with water, in a volumetric ratio of 1:100,000. To determine the LC50, tanks were filled with 2.5, 3, 3.5 and 4.0 mL L^{-1} of diluted chlorpyrifos, and treated snails were compared with a control group that had no chlorpyrifos added. In each treatment, 10 snails were kept in the tanks throughout the experimental period without feeding. The cumulative mortality rates were recorded at 0, 24, 48, 72 and 96 h and the LC50 of chlorpyrifos was evaluated with Probit analysis by using the Minitab® 17 software (entitlement i.d.: 2ec6-9b37-1508-0264-2c55-c33).

Sublethal toxicity assay for tissue preparation of AChE expression. Morphological changes were observed on *P. canaliculata* at 24, 48, 72 and 96 h of exposure and compared with the control group. Then, after the lethal concentration of chlorpyrifos was identified, concentrations below that level were chosen for the study on the AChE expression response of snail. The amounts of chlorpyrifos used were 0, 0.5, 1.0, 1.5 and 2.0 mL L^{-1} with survival measured at 0, 24, 48, 72 and 96 h (triplicates performed for each treatment). The tissue preparation for measuring AChE expression was modified from Thanomsit et al (2017); twenty snails were used for each treatment. For sampling, the snails (n=3) were collected, washed and then placed in an ice box. Then, *P. canaliculata* flesh was removed from the shell. The tissue was blended in a homogenization buffer (50 mM Tris-HCl pH 7.2, containing 0.1% triton X-100 and 0.1% NaCl). The homogenate was centrifuged at 350 rpm for 65 min at 4ºC. The supernatant was immediately frozen until use.

Protein determination. The supernatant of protein samples was diluted in 0.02 M phosphate buffer saline (PBS) at pH 7.2 and at the ratio of 1:50. A standard curve of bovine serum albumin (BSA) was plotted based on protein concentration. The assay used a UV-visible spectrophotometer (Thermo Scientific Co., Ltd., model GENEYS 10S UV-VIS) and was performed in triplicate. A standard curve of absorbance at 280 nm (Y axis) against Bovine serum albumin (BSA) concentration (mg mL^{-1}) (X axis) was then plotted. The standard curve was used to determine the protein concentration of all samples.

Acetylcholinesterase (AChE) expression evaluated by western blot. Supernatant containing AChE was diluted with PBS and then mixed with 2x buffer at the concentration of 4 µg μL^{-1}. Then, 10 µL of the prepared sample was loaded in the protein lanes. After that, the protein was separated by electrophoresis in 10% sodium dodecyl sulfate

polyacrylamide gel (10% SDS-PAGE), stained by Coomassie Brilliant Blue R-250 and then a western blot analysis was performed using a protocol slightly modified from Thanomsit et al (2018). In brief, the protein was transferred to cellulose membrane and incubated in 5% skim milk in PBS for 1 h. Next, the excess was washed off and the membrane was soaked in Polyclonal antibody specific to AChE from electric eel (commercial polyclonal antibody specific to acetylcholinesterase; PAb-AChE), obtained from Raybiotech© at a dilution of 1:200 for 12 h. The membrane was washed using PBS-Tween 20 (PBS-T) and then incubated in the secondary antibody labelled with the enzyme (GAR-HRP dilution 1:1,000; catalog number # ab 6741) for 3 h. It was then washed by PBS-T for 5 min 3 times. Finally, it was incubated in substrate solution (0.03% diaminobenzidine (DAB), 0.006% H_2O_2 and 0.05% $CoCl_2$ in PBS) and checked for the presence of AChE.

Dot blot analysis. Protein sample was prepared to a concentration of 4 μg μL^{-1}. After that, the sample was dotted onto nitrocellulose membrane and left to dry for approximately 1 h. Next, the membrane was incubated in 5% skim milk in PBS for 1 h. Then, it was washed with PBS-Tween for 5 min (3 times). It was then incubated with primary antibody (polyclonal antibody specific to AChE; PAb-AChE) specific to electric eel at the dilution of 1:200 for 12 h. Next, it was washed with PBS-T 3 times and then incubated in secondary antibody (Goat anti-Mouse antibodies conjugated with horseradish peroxidase; GAR-HRP dilution of 1:2,000) for 3 h. The excess antibody was washed off. Next, it was soaked in a substrate solution for 3 min. A positive result indicating AChE was a dark brown dot.

Immunohistochemistry analysis. The intestinal tissue of *P. canaliculata*from the non-treated group and chlorpyrifos treated groups (concentrations of 0.5, 1.0, 1.5 and 2 mL L^{-1} for 24, 48, 72 and 96 h) were sliced into small pieces and fixed with 10% phosphate buffer formalin solution for 24 h. Next, the tissues were dehydrated in a series of ethanol washes (50, 70, 80 and 90%, and absolute ethanol), then cleared in xylene, infiltrated and embedded in paraffin. The blocks of specimen were sectioned to a thickness of 6 μm by using a microtome. For antibody testing, the slide was stained by addition of $P1^+$ (10% fetal calf serum in PBS pH 7.2) on the tissue slide and incubation for 30 min. After that, it was further incubated with primary antibody specific to AChE (commercial polyclonal antibody) at a dilution of 1:200 for 3 h. Next, the slide was soaked in PBS for 10 min 4 times. Then, it was incubated with GAR-HRP 1:1,000 at 37°C for 3 h and soaked in PBS for 10 min 3 times. At room temperature, 3'3'diaminobenzidine (DAB) was added to the sectioned organ, followed by further incubation for 10 min in the dark. The slide was washed 3 times with reagent quality water and then counterstained with Mayer's hematoxylin and eosin (H&E) for 2 min and washed in running water. Gradient ethanol was used to dehydrate the section, which was further mounted using permount. The control was treated with $P1^+$ instead of antiserum. The slides were studied under a microscopic.

Detection of AChE expression in golden apple snail from from Huai-Saneng Reservoir. Golden apple snails were collected from 3 sampling stations (n=3) in Huai-Saneng reservoir, Surin province in January, 2020 to interrogate AChE expression. The geographic coordinates for each sampling station of collected snail and water samples for organic organophosphate analysis were 14.815071 N, 103.5030444 E (station 1), 14.807894 N, 103.510465 E (station 2) and 14.808923 N, 103.504683 E (station 3) (Figure 1). To measure organophosphate concentration in the water, we used a GT-Pesticide Test Kit (accuracy 94%), purchased from Higher Enterprises Co., Ltd. Water sample was collected from each station where *P. canaliculata* were collected. Phenotypic characterization of each collected golden apple snail was recorded. After that, protein was extracted to identify AChE expression using western blot and immunohistochemistry techniques, as applied in the laboratory studies.

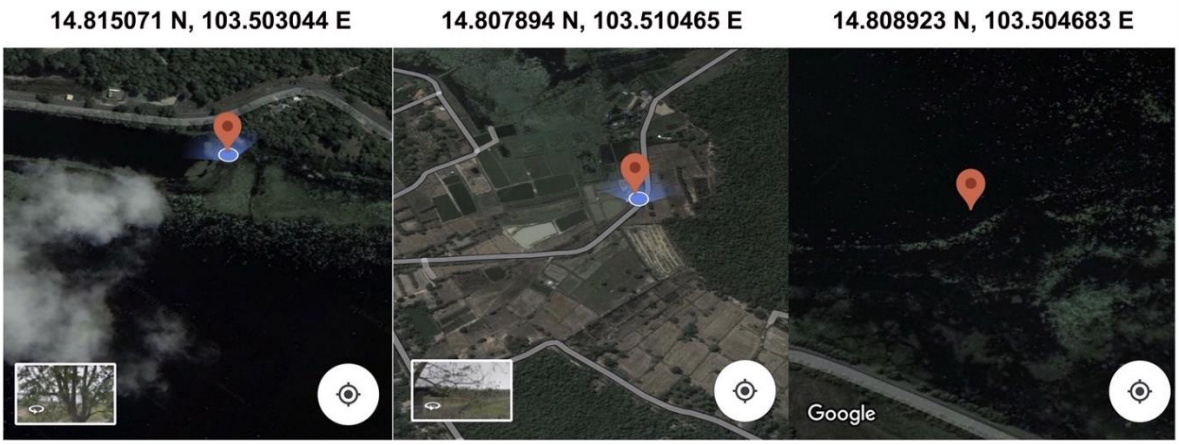

Figure 1. Sampling stations to collect *Pomacea canaliculata* and water samples in Huai-Saneng reservoir, Surin province, Thailand.

Results and Discussion. Water pollution is one of the main environmental problems in Thailand. Specifically, in the agricultural sector, pesticide use is extensive and increasing. In addition, in the case of excessive or non-appropriate application, pesticides may cause adverse effects on human-beings or the environment. Many studies revealed that surface waters are contaminated with insecticides, resulting in water quality degradation and health risks to aquatic organisms. For example, Basopo & Ngabaza (2015) reported that aquatic snail (*Helisoma duryi*) was exposed to chlorpyrifos and lead in a reservoir, and was then used as a bioindicator. Besides, Ran et al (2020) claimed that *Lymnaea stagnalis* was applied as a bioindicator for insecticide contamination, e.g. chlorpyrifos and cypermethrin, in freshwaters with its life cycle. Importantly, contamination in edible and/or economic aquatic animals can cause adverse health effects to humans, as the top consumer, or result in trade rejection from international trading partners (Mararam et al 2016). Chlorpyrifos is an insecticide in the organophosphorus group that causes both systematic and non-systematic effects.

Chlorpyrifos has a phosphorous in its structure and can eliminate a wide variety of insects. Generally, it is readily available and cheap. Moreover, it is rapidly degraded compared to other pesticides; consequently, it is widely used in agriculture. It affects the nervous system by inhibiting the function of AChE causing ACh accumulation in the nerve endings. This phenomenon causes uncontrolled body movement, convulsion, dribbling, and urine droplets. It can be assimilated into the body by feeding and environmental exposure. In the case of high dose, it can cause acute effects (Petchoy & Pung 2017). Prasopsuk et al (2014) studied the types of insecticide found in vegetables and fruits collected from the Eastern-part Good Manufacturing Practice (GMP) certified farms during 2011-2013. They found that the most common insecticides were chlorpyrifos, cypermethrin, methomyl and carbaryl. In Thailand, chlorpyrifos has not been officially banned, and is used without consideration of international guidelines. Thus, in this study we aimed to evaluate the toxicity level of chlorpyrifos in golden apply snail so that the results can be used in that decision making process. We chose golden apple snail because it can be widely found and is a food source in some areas, thus contamination may cause a health risk.

The measurement of AChE activity can be used as biomarker of exposure for insecticides. In the case of long exposure time and high concentration, AChE will be degraded and the tissue is altered (Putkome et al 2008). However, in the case of short exposure time and low concentration, the organism is induced to synthesize AChE more than under normal conditions. Thus, many studies suggested that AChE could be applied as biomarker. Walker et al (2006) showed that AChE is a biomarker specific to insecticides in the organophosphate and carbamate groups.

Morphological changes. In this study, it was found that many morphological changes occurred in *P. canaliculata* after exposure to chlorpyrifos compared with those in the

control group (Figure 2A and B). The snails exposed to chlorpyrifos at sublethal-acute-toxicity levels expressed darker color in the head, body and digestive tract than those of the control group after 24-48 h of exposure (Figure 3). Thanomsit et al (2017) reported that morphological alterations could be used in assessing insecticide exposure in both pond snail and *P. canaliculata*.

Based on this study, it is possible to use morphology to identify chlorpyrifos exposure in *P. canaliculata*. This information can reduce the risk to people in Northern Thailand that prefer to eat *P. canaliculata*, allowing them to visually identify contaminated snails at the point of collection. Moreover, *P. canaliculata* is an important protein source for many animals, such as quail, and is used in the development of feed for rice-field crabs (Phumrojana et al 2018; Tanee et al 2015). These animals are consumed by humans, so there is a possibility of exposure to chlorpyrifos. Therefore, the study of morphological changes is important.

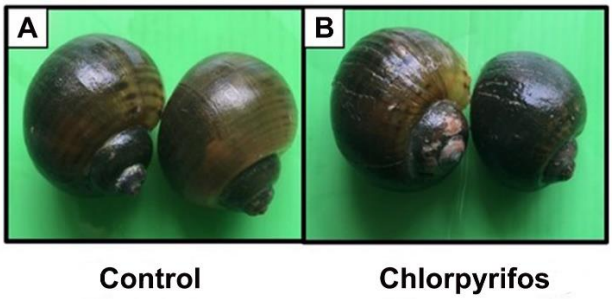

Figure 2. External morphological appearances of *Pomacea canaliculata* exposed to chlorpyrifos (B) and control group (A).

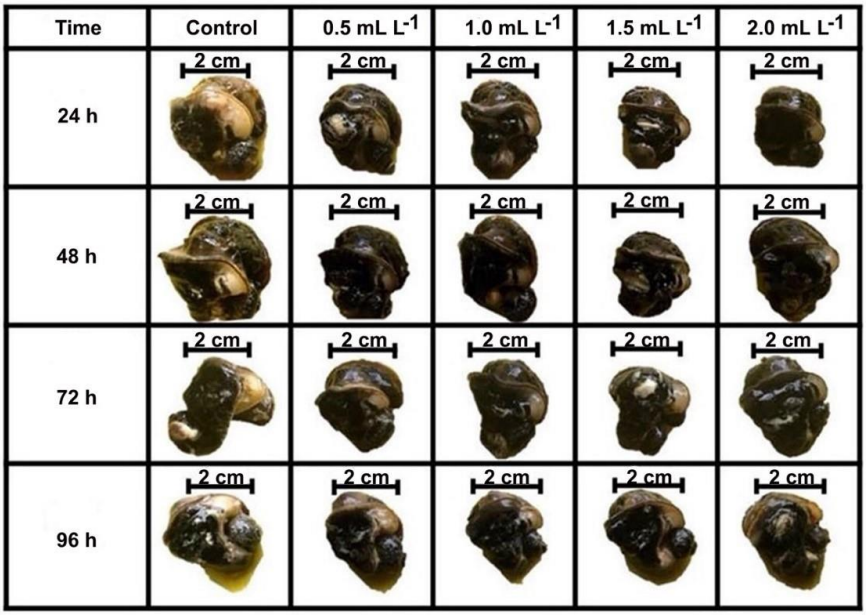

Figure 3. Characteristics of tissue of *Pomacea canaliculata* exposed to chlorpyrifos compared to control for 24, 48, 72 and 96 h.

Acute toxicity evaluation by Probit analysis. Laboratory bioassays are important in generating toxicity data of various chemicals in short times with low cost. It is a simple, versatile, easy and sensitive technique for determining toxicity of a wide range of chemicals, which greatly facilitates the determination of the LD_{50}, LC_{50} or any other lethal concentration/dose (Paramasivam & Selvi 2017). The laboratory bioassays reveal information of the interactions of insect-insecticide or insect-plant-insecticide. The objective of the present work was to employ laboratory bioassay techniques to evaluate the toxicity of chlorpyrifos. The accumulative mortality of *P. canaliculata* increased with an increase in exposure time and concentration. At first, chlorpyrifos was diluted with dI

water in the ratio of 1:100,000 (volume by volume) and then the solution was mixed with water in the ratio of 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4.0 mL L^{-1}. *P. canaliculata* was placed in the experimental tank for 24, 48, 72 and 96 h, to identify lethal and sublethal concentrations. We found that sub-lethal concentrations were 0.5, 1, 1.5 and 2.0 mL L^{-1}. There were 4 levels of concentration that showed acute toxicity and mortality higher than the control group: 2.5, 3, 3.5 and 4.0 mL L^{-1}. The lowest concentration causing mortality was 2.5 mL L^{-1} after 24 h of exposure and the mortality percentage was 15.8%. The highest cumulative mortality was found in the concentration of 4 mL L^{-1} as shown in Figure 4.

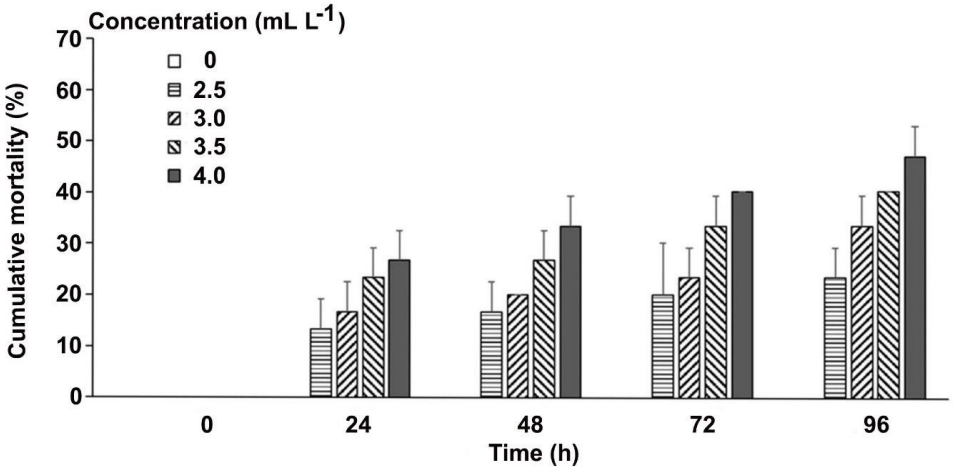

Figure 4. Cumulative mortality percentage of *Pomacea canaliculata* exposed to chlorpyrifos at concentrations of 2.5, 3.0, 3.5 and 4.0 mL L^{-1} for 24, 48, 72 and 96 h compared with control group.

In this study, the dose-response relationship was evaluated and it was found that the no-observed-adverse-effect-level (NOAEL) and the lowest-observed-adverse-effect (LOAEL) level were log concentrations of 3.0 mL L^{-1} and 4.0 mL L^{-1} of chlorpyrifos in all exposure times (Figure 5). Probit analysis was performed to assess the LC_{50}. At 24 h of exposure, the LC_{50} was 7.00 (6.02-8.93) mL L^{-1}, after 48 h, 5.91 (5.29-6.97) mL L^{-1}, whereas after 72 h the LC_{50} was 4.90 (4.58-5.41) mL L^{-1}. Finally, after 96 h of exposure, the LC_{50} was 4.22 (4.02-4.50) mL L^{-1}.

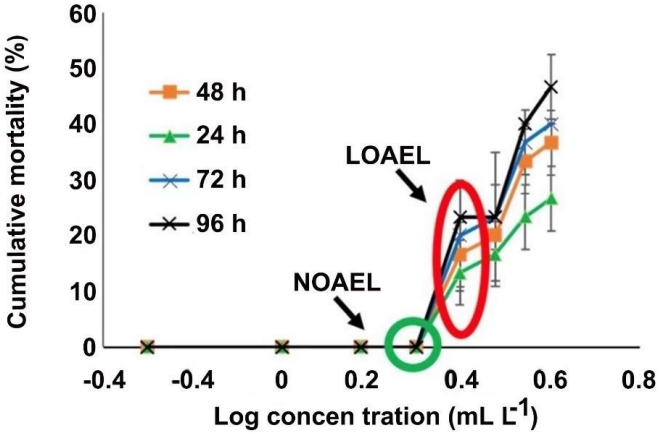

Figure 5. Dose-response relationship of chlorpyrifos concentration and the cumulative mortality percentage on *Pomacea canaliculata*.

AChE expression and western blot analysis. Protein extracted from apple snail, exposed to chlorpyrifos at sublethal concentrations, was separated using 10% SDS-PAGE (Figure 6A). We found AChE expression from the control and all chlorpyrifos exposure times at three concentrations: 0.5, 1.0 and 1.5 mL L^{-1}. The results of western blot

revealed dark brown protein bands of AChE with molecular weight of 71 kDa (Figure 6B-D). For the concentration of 2.0 mL L^{-1}, AChE expression was found at only 24 and 48 h after exposure (Figure 6E). This finding suggests that chlorpyrifos in high concentrations might destroy nervous system tissue resulting in no AChE expression at 72 and 96 h. In addition, the long exposure time (96 h) had lower AChE expression compared to the control and 24 h exposure groups.

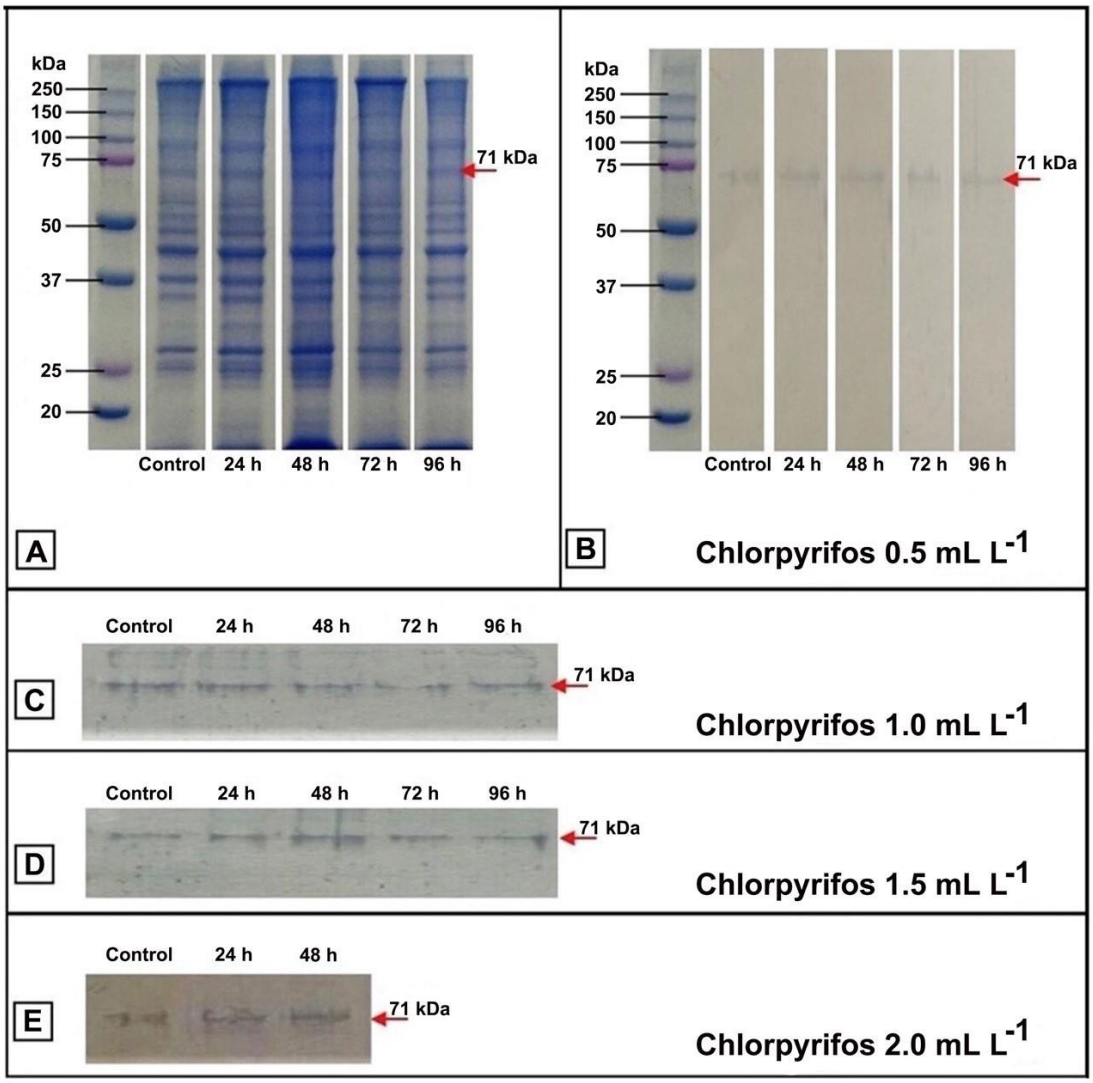

Figure 6. 10% SDS-PAGE expressed pattern of protein extracted from *Pomacea canaliculata* exposed to chlorpyrifos in the concentration of 0.5 mL L^{-1} at 24, 48, 72 and 96 h after exposure compared to the control (A). Western blot analysis for specificity of AChE from golden apple snail exposed to chlorpyrifos in the concentration of 0.5 mL L^{-1} (B) 1.0 mL L^{-1} (C) 1.5 mL L^{-1} (D) and 2.0 mL L^{-1} (E).

There is a lack of information relevant to AChE in snail; however, some reports indicated that AChE could be used as a biomarker of insecticide exposure. Chitmanat et al (2008) used AChE from river snail (*Sinotala ingallsiana*) as biomarker to assess insecticide exposure in the Upper Ping River, which is an area nearby intensive agriculture. They found that AChE expression in three studied areas was lower thanat the reference site. They concluded that AChE could be used as biomarker of pesticide contamination. In 2006, Gaitonde et al (2006) studied AChE activity in marine snail (*Cronia contracta*) to assess contaminant in West coast of India. They found that AChE from contaminated areas was lower than that from other areas and reference sites. Our results are in agreement with the study of Thanomsit et al (2017) who reported AChE expression in pond snail and golden apple snail collected from local markets in Surin

province. The positive result indicated by a dark brown band of a protein with the molecular weight of 71 kDa, which was different from the study of Ma et al (2011). The latter found that pure AChE in *Pardosa astrigena* (L. Koch) had the molecular weight of 66.35 kDa. This difference might be caused by distinct measurement techniques.

Dot blot was applied to assess AChE expression. Dot blot was used to assess AChE expression because many studies indicated that it is rapid and highly specific. Moreover, it can be applied in testing a large number of samples. In this study, dot blot showed the expression of AChE at 0, 24, 48, 72 and 96 h at concentrations of 0.5 and 1.0 mL L^{-1}. At concentrations of 1.5 and 2.0 mL L^{-1}, AChE was only detected at 24, 48, and 72 h of exposure. The decrease of AChE expression at high exposure times and concentrations might be because chlorpyrifos kills the cells that produce AChE (Figure 7).

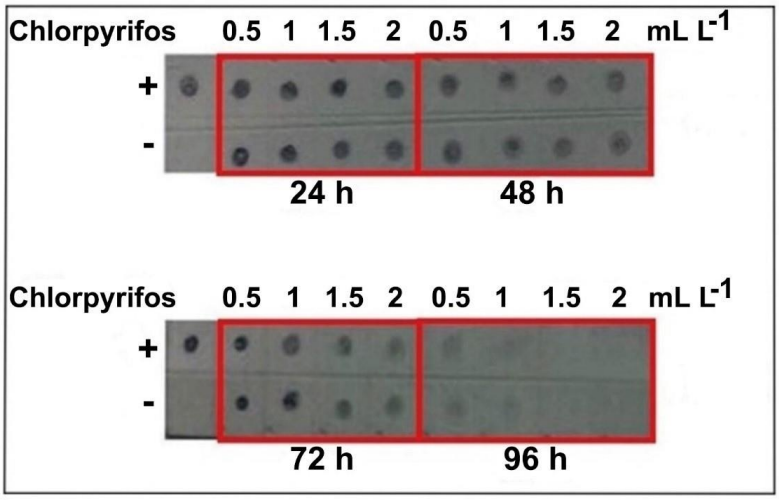

Figure 7. Dot blot for AChE expression in *Pomacea canaliculata* after exposure to chlorpyrifos at concentrations of 0.5, 1.0, 1.5 and 2.0 mL L^{-1} and exposure times of 24, 48, 72 and 96 h. Positive control (+): AChE from hybrid catfish; Negative control (-): vitellogenin from Nile tilapia.

Localization of AChE using immunohistochemistry. Immunohistochemistry is an interesting technique based on the principle of antibodies binding to specific antigens (Guzmán-Guillén et al 2013). It is generally used in toxicological testing and aquatic disease testing because it identifies presence and cellular localization in organisms (Thanomsit et al 2021). Otludil et al (2004) reported that sublethal concentrations caused adverse effects in the digestive gland, foot and mantle of the great ramshorn snail in small ponds and streams. In addition, Dreon et al (2003) also studied the effect of egg perivitellin fluid (PVF) administration on rat small intestine cells, cell proliferation and glycosylation pattern by immunohistochemistry. They found that antibody could cross-react and gave a clear positive result in many areas, such as the basal zone of the epithelium. The present study revealed that chlorpyrifos caused histological alterations in the intestine of golden apple snail. Moreover, immunohistochemistry results showed moderate and strong AChE stain in the intestine of control snails and in snails treated with chlorpyrifos for 24 to 72 h. However, chlorpyrifos at the concentration of 0.5 mL L^{-1} caused a decrease in AChE after 96 h of exposure. In the control, localization of AChE was detected in muscular layer, nucleus, goblet cell, and mucous secreting cells (Figure 8A). After the exposure of the golden apple snail to chlorpyrifos, the alterations found were edema and disorganization of mucosa and sub-mucosa. The alterations became more pronounced with an increasing exposure time and concentration (Figure 8 and Table 1). There were some reports indicating that chlorpyrifos could destroy tissues, causing many histological changes in the digestive gland of *H. vestalis* after exposure to sub-lethal concentrations of both methiocarb and chlorpyrifos pesticides. These alterations included severe tubular disruption, vacuolation, pyknotic nuclei, and necrosis of digestive tubules (Sharaf et al 2013).

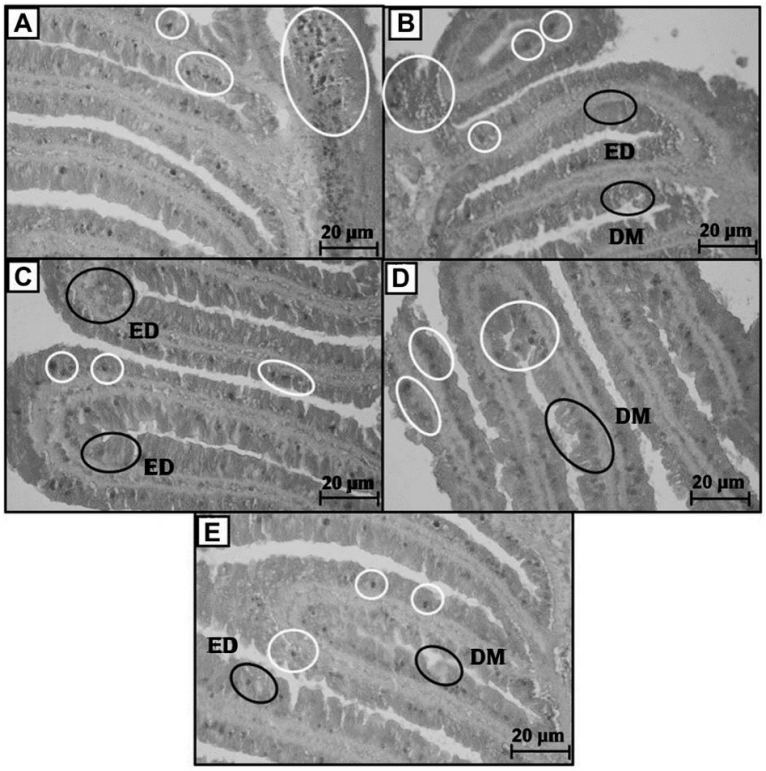

Figure 8. Example of immunohistochemistry results in *Pomacea canaliculata* intestine exposed to chlorpyrifos at 0.5 mL L^{-1}, stained with PAb-AChE specific to electric eel diluted to 1:200. Control group (A), 24 h exposure (B), 48 h exposure (C), 72 h exposure (D), and 96 h exposure (E). White circles highlight localization of AChE expression stained with PAb-AChE (commercial polyclonal antibody), and black circles highlight tissue alteration, ED: edema and DM: disorganization of mucosa and sub mucosa.

Table 1

Immunohistochemistry and histological alterations of intestine of *Pomacea canaliculata* exposed to chlorpyrifos at the sub-lethal concentrations of 0, 0.5, 1.0, 1.5 and 2.0 mL L^{-1} for 24, 48, 72 and 96 h

Time (h)	*Concentration (mL L^{-1})*	*Histological alterations*		*Immunohistochemistry (%)*
		Edema	*Disorganization of mucosa and sub-mucosa*	
24	0	-	-	100
	0.5	+	-	100
	1.0	+	-	100
	1.5	+	+	100
	2.0	++	++	100
48	0	-	-	100
	0.5	+	-	100
	1.0	++	+	100
	1.5	++	++	100
	2.0	++	++	100
72	0	-	-	100
	0.5	+	+	90
	1.0	++	++	90
	1.5	++	++	No
	2.0	+++	+++	No

96	0	-	-	100
	0.5	+	+	80
	1.0	++	++	80
	1.5	+++	+++	No
	2.0	+++	++++	No

No change (-); small change (+); medium change (++); abundant change (+++); No-not detected.

Study of AChE expression in Huai-Saneng Reservoir, Surin province. The study of pesticide contamination in aquatic environments and organisms, especially in snail, are still quite rare. If consumed, pesticides can cause adverse health effects. There are many ways to detect contamination from these substances, but it is widely accepted that the use of AChE expression is appropriate, as a specific biomarker of these substances. Chitmanat et al (2008) reported a significant contamination of organophosphate and carbamate pesticide in river snail (*Sinotalia ingallsiana*), due to dense agriculture nearby the Ping River in Chiangmai province,by detection of the AChE as a biomarker. The decreased AChE expression found was lower than in the control group, in every season. Moreover, Thanomsit et al (2018) evaluated the pesticide exposure in egg and tissue of *P. canaliculata* that was collected from Huai-Saneng Reservoir, Surin province by using AChE as biomarker. The AChE expression in snails from stations with the highest organochlorine and carbamate pesticide contamination were lower than at the other stations. However, community expansion caused the environment to change, requiring further research. In this study, the expression of AChE was measured in the samples from Huai-Saneng Reservoir because it is an important reservoir for the community. The samples were collected and studied in January 2020. We found organophosphorus pesticide contamination in the water (tested by GT-Pesticide Test Kit) at stations 2 and 3. The morphological appearance and tissue of snails from stations 2 and 3 were abnormal, and the expression of AChE (tested using the western blot and immunohistochemistry) was lower at station 1. This may be caused by organophosphate insecticides damaging the tissues and cells, resulting in less expression of AChE (Table 2).

Table 2

Acetylcholinesterase on *Pomacea canaliculata* from Huai-Saneng reservoir, Surin province

Station	*Replicates (n=5)*	*Morphology*	*Western blot (71 kDa)*	*Immmunohistochemistry (%)*	*GT-Pesticide test kit*
Station 1 (14.815071, 103.5030444) near communities	1	Shell was dark brown, no cracks, normal tissue	+	+ (100)	-
	2	Shell was dark brown, normal tissue	+	+ (100)	-
	3	Shell was dark brown, normal tissue	+	+ (100)	-
Station 2 (14.807894, 103.510465) near rice fields	1	Shell was dark brown, normal tissue	+	+ (90)	+
	2	Shell was dark brown, tissue was pale	+	+ (60)	+
	3	Shell was dark brown and tissue was decayed	–	-	+

Station	*Replicates (n=5)*	*Morphology*	*Western blot (71 kDa)*	*Immmunohisto chemistry (%)*	*GT-Pesticide test kit*
Station 3 (14.808923,103.50468) near fish pond	1	Shell was dark, tissue was pale	+	+ (60)	+
	2	Shell was dark brown and tissue was decayed	-	-	+
	3	Shell was dark, tissue was pale	+	+ (20)	+

+ Found interaction / positive result; - Not found interaction / negative result.

Based on our results, we found that chlorpyrifos had negative effects on the golden apple snail. Initially, an assessment of the toxicity and morphological change is important to avoid consumers' exposure, by identifying contaminated snails through fast techniques. From the results of this study, the immunological techniques such as dot blot, western blot and immunohistochemistry can be used to assess the exposure of chlorpyrifos. Concentration and exposure time should be considered in the assessment of AChE expression to indicate chlorpyrifos exposure.

Conclusions. Chlorpyrifos is an insecticide, which can cause adverse effects in *P. canaliculata*. The study showed that tissue of exposed snail was darker than in the control. Moreover, it was also found that cumulative mortality increased with an increasing exposure time and concentration. The molecular weight of AChE was 71 kDa, measured using dot blot and Western blot. The expression of AChE decreased with an increasing chlorpyrifos exposure time and concentration. Immunohistochemistry clearly identified the localization of AChE into the intestine. The alterations in the intestine were edema and disorganization of mucosa and sub-mucosa. Based on the current findings, it can be concluded that chlorpyrifos caused adverse snail tissue changes. The expression of AChE can be used as an early warning signal in assessing chlorpyrifos exposure at sub-lethal levels. Moreover, AChE expression can be used in measuring organophosphate exposure in *P. canaliculata* in aquatic environments. The results can also be used as fundamental knowledge to assess chlorpyrifos contamination in wild snails and to reduce health risk in the consumer, by providing phenotype information that indicates contamination.

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